ESTABLISHMENT OF ADVENTITIOUS ROOT CULTURE OF BUTTERFLY PEA PLANT (*Clitoria ternatea* L.) AND ITS POTENTIAL AS A MEMORY ENHANCER

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ESTABLISHMENT OF ADVENTITIOUS ROOT CULTURE OF BUTTERFLY PEA PLANT (*Clitoria ternatea* L.) AND ITS POTENTIAL AS A MEMORY ENHANCER

by

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LIST OF ABBREVIATIONS

½ MS	Half strength Murashige and Skoog basal medium
2,4-D	2,4-dichlorophenoxyacetic acid
2-iP	2-isopentenyl adenine
4-Cl-IAA	4-chloroindole-3-acetic acid
AChE	Acetylcholinesterase
ANOVA	Analysis of variance
ATCI	Acetylthiocholine iodide
b.w.	Body weight
B5	Gamborg's B5 basal medium
BAP/BA	6-benzylamino purine
C	Cortex
CTR	*C. ternatea* root extract
DKW	Driver and Kuniyuki basal medium
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
ECM	Extracellular matrix
ECT	Ethanolic extract of *C. ternatea* L. outdoor-grown plant root
ECTN	Ethanolic extract of *C. ternatea* L. *in vitro* NAA-induced root
ECTS	Ethanolic extract of *C. ternatea* L. *in vitro* seedling root
FL	Fibrillar layer
G	Granular structure
GC-MS	Gas chromatography-mass spectrometry
HPTLC	High-performance thin-layer chromatography

i.p.	Intraperitoneal injection
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IC_{50}	Half maximal inhibitory concentration
IPA	indole-3-propionic acid
JA	Jasmonic acid
Kin	Kinetin
MAO	Monoamine oxidase
MeJA	Methyl jasmonate
MW	Molecular weight
NAA	1-naphthaleneacetic acid
p.o.	Per os. or oral administration
P	Phloem
PGRs	Plant growth regulators
SA	Salicylic acid
SE	Standard error
SEM	Scanning Electron Microscopy
w/v	Weight per volume
X	Xylem
ZR	Zeatin riboside

PENUBUHAN KULTUR AKAR ADVENTITIUS BUNGA TELANG (*Clitoria ternatea* L.) DAN POTENSINYA SEBAGAI PENGGALAK INGATAN

ABSTRAK

Clitoria ternatea L., biasanya dikenali sebagai bunga telang ialah tumbuhan herba kembaran perenial dari keluarga Fabaceae yang berasal dari Asia tropika. Tumbuhan ini adalah ubat herba tradisional terkenal yang diiktiraf sebagai Aparajita atau Shankhpushpi oleh sistem perubatan Ayurveda kerana kesan ubat-ubatan yang meluas seperti penggalak kognitif, anti-sawan dan anti-diabetes. Ia secara tradisinya dikenali sebagai tonik otak dan telah dikaji secara meluas kerana mempunyai keupayaan sebagai penggalak ingatan atau rawatan alternative kepada penyakit Alzheimer. Kajian semasa bertujuan untuk menyiasat kesan pelbagai auksin dalam penubuhan kultur akar *in vitro* daripada eksplan kotiledon, hipokotil, nod kotiledon dan akar *C. ternatea* L. dan untuk mengenal pasti kehadiran sebatian-sebatian bioaktif dalam kultur akar yang berkaitan dengan sifat penggalak ingatan. Empat jenis eksplan ini diperoleh daripada anak benih *in vitro* berumur 2 minggu dan dikulturkan pada medium MS separuh kepekatan (½ MS) yang ditambah dengan lima jenis auksin berlainan kepekatan iaitu NAA, IBA, IAA, 4-Cl-IAA dan 2,4-D. Kesan penambahan lima jenis auksin ini pada pengaruhan kultur akar adventitius secara *in vitro* adalah bergantung kepada jenis eksplan. Peratusan pengaruhan kultur akar tertinggi pada 95.24% telah berjaya dicapai oleh eksplan kotiledon yang dikulturkan di atas medium ½ MS diperkaya dengan 2.5 mg/L NAA. Sebaliknya, penambahan 1.0 dan 1.5 mg/L IAA telah menghasilkan peratusan pengaruhan akar sebanyak 83.33% daripada eksplan hipokotil. Bilangan akar adventitius yang lebih tinggi dihasilkan daripada

eksplan kotiledon (12.86±2.14) dan eksplan hipokotil (8.67±2.73) apabila masing-masing dikulturkan atas medium diperkaya dengan 1.5 mg/L NAA dan 2.0 mg/L IAA. Sementara itu, panjang maksimum akar adventitius dihasilkan oleh eksplan kotiledon dengan penambahan 2.5 mg/L NAA (0.64 ± 0.04 cm) dan eksplan hipokotil dengan penambahan 1.0 mg/L IBA (0.79±0.10 cm). Dalam kajian ini, jelas bahawa pengaruhan akar daripada eskplan kotiledon yang dikulturkan di atas medium ½ MS dengan penambahan NAA diukur dengan diameter yang lebih tebal (1.9 dan 2 kali ganda). Pembentukan kalus oleh eksplan kotiledon dikesan apabila diinokulasi pada medium ½ MS diperkaya dengan 2,4-D. Perencatan aktiviti acetylcholinesterase (AChE) oleh ekstrak akar adventitius secara *in vitro* hasilan pengaruhan NAA yang dikaji menggunakan kaedah Ellman menunjukkan keupayaannya dalam penggalakkan daya ingatan walaupun lebih rendah daripade ekstrak akar tumbuhan liar. Keputusan analisis GC-MS mengenal pasti kehadiran triterpenoid pentasiklik dalam kultur akar secara *in vitro*. Kajian ini telah berjaya menubuhkan kultur akar *C. ternatea* L. secara *in vitro* daripada eksplan kotiledon dan ekstraknya mengandungi sebatian-sebatian bioaktif seperti triterpenoid pentasiklik, fitosterol dan asid lemak yang dapat dikaitkan dengan potensinya sebagai penguat daya ingatan. Ini menunjukkan bahawa kultur akar *in vitro* adalah alternatif yang berpotensi dan pengeluaran metabolit sekunder boleh dikaji dengan lebih lanjut sebagai satu sumber yang mampan untuk penghasilan sebatian-sebatian perubatan yang berharga bagi industri farmaseutikal terutamanya berkaitan dengan penyakit neurodegeneratif.

ESTABLISHMENT OF ADVENTITIOUS ROOT CULTURE OF BUTTERFLY PEA PLANT (*Clitoria ternatea* L.) AND ITS POTENTIAL AS A MEMORY ENHANCER

ABSTRACT

Clitoria ternatea L., commonly known as Butterfly Pea or bunga telang, is a perennial twining herbaceous plant from the Fabaceae family, native to tropical equatorial Asia. This twinning herb is a well-known traditional herbal medicine recognized as Aparajita or Shankhpushpi by the ancient Ayurvedic medicine system owing to its outstanding medicinal effects as nootropic, anti-convulsant and anti-diabetic. It is traditionally known as a brain tonic and has been widely studied for its memory booster potential or an alternative treatment for Alzheimer's. The current study aims to investigate the effects of various auxins in the establishment of *in vitro* root culture from cotyledon, hypocotyl, cotyledonary node and root explants of *C. ternatea* L. and to identify the presence of bioactive compounds from the root cultures correlated to memory enhancing properties. The four types of explants were obtained from 2-weeks-old *in vitro* seedlings and cultured on semi-solid ½ MS medium supplemented with different concentrations of NAA, IBA, IAA, 4-Cl-IAA and 2,4-D. The effects of auxins on *in vitro* adventitious root induction was explant type-dependent. The highest root induction percentage (95.24%) was successfully induced using cotyledon explants in ½ MS medium fortified with 2.5 mg/L NAA. In contrast, supplementation of 1.0 and 1.5 mg/L IAA resulted in higher root induction percentage for hypocotyl explants (83.33%). The relatively higher adventitious root number were induced from cotyledon (12.86±2.14) and hypocotyl (8.67±2.73) explants at the supplementation of 1.5 mg/L NAA and 2.0 mg/L IAA respectively. Meanwhile, the

maximum adventitious root length was induced from media supplemented with 2.5 mg/L NAA and 1.0 mg/L IBA for cotyledon (0.64±0.04 cm) and hypocotyl (0.79±0.10 cm) explants respectively. In the present study, it was evident that roots induced on half strength MS media with NAA were abnormally wider in diameter (1.9- and 2-fold). Callus formation was observed for cotyledon explants inoculated with 2,4-D. The anti-acetylcholinesterase (AChE) potential of *in vitro* NAA-generated root extracts determined using Ellman's method showed that AChE activity was inhibited, indicating potential memory enhancing ability albeit lower than the effect of outdoor-grown plant. GC-MS analysis of the *in vitro* NAA-root extract identified the presence of pentacyclic triterpenes in the root cultures. The current study has successfully established *in vitro* root cultures of *C. ternatea* L. from cotyledon explants possessing pentacyclic triterpenes, phytosterols and fatty acids that are potential bioactive compounds linked to memory enhancing properties. This indicated that the *in vitro* adventitious root culture is a potential alternative that can be further investigated in the production of secondary metabolites as a sustainable source for valuable medicinal compounds for pharmaceutical industry especially linked to neurodegenerative diseases.

CHAPTER 1

INTRODUCTION

Butterfly pea, or scientifically known as *Clitoria ternatea* L. is a perennial herbaceous climber belongs to Fabaceae family that is native to Africa and widely distributed in India, Caribbean, Philippines, Australia as well as Malaysia (Mukherjee *et al.*, 2008; Gupta *et al.*, 2010). Some other common names of *C. ternatea* include Aparajita, Cordofan pea, blue pea and Asian pigeonwings. In Malaysia, the flower and leaves of *C. ternatea* is traditionally incorporated in foods like Pulut Tai Tai, Pulut inti and Nasi Kelabu (Mukherjee *et al.*, 2008). Though the plant exhibit vigorous growth in rough conditions, it was found to have low seed germination frequency and yet high mortality rate of juvenile seedlings (Singh & Tiwari, 2010). Such conditions have hastened the need of establishment of proper and efficient micropropagation protocol for *C. ternatea* to ensure the reproducibility and survival of the species.

C. ternatea is known for its wide range of medicinal properties and is recorded in the Ayurvedic medicine system as brain tonic (Mukherjee *et al.*, 2008; Dighe *et al.*, 2009; Al-Snafi, 2016). Also called Shankhpushpi or Aparajita in the Indian traditional medicine, its applications include anti-inflammatory, antioxidant, anti-depression, anti-diabetic, analgesic, hepatoprotective as well as nootropic and promoting intelligence (Mukherjee et al., 2008; Gupta *et al.*, 2010; Al-Snafi, 2016; Mehla & Gupta, 2018; Muhammad & Rabeta, 2018). Various bioactive compounds identified from different parts of the plants which include saponins, flavonoids, pentacyclic triterpenoids, ternatins and anthocyanins (Terahara *et al.*, 1996; Gomez & Kalamani, 2003; Al-Snafi, 2016).

Plants are major sources for various valuable medicinal secondary metabolites that are important for pharmaceutical and nutraceutical developments (Flores *et al.*, 1987; Rao & Ravishankar, 2002). The leaf extract of *C. ternatea* was reported with anti-oxidative and hepatoprotective properties which might be associated with the steroids, flanonoids, phenols and tannins constituents in the leaf extract (Nithianantham *et al.*, 2011; Kavitha & Premalakshmi, 2013). The flowers of the plant were also identified with the presence of anthocyanins, ternatin and flavonols with potential anti-inflammatory effect (Terahara *et al.*, 1996; Nair *et al.*, 2015). In addition, Arumugam and Panneerselvam (2012) reported that the methanolic extract of *in vitro* derived plant and callus of *C. ternatea* has shown significant antibacterial activity toward certain pathogenic bacteria owing to its high isoflavonoid content.

The root of *C. ternatea* contains a variety of secondary metabolites such as tannins, alkaloids, flavonoids, glycosides and pentacyclic triterpenoids (Mukherjee *et al.*, 2008; Gupta *et al.*, 2010; Chauhan *et al.*, 2012b; Swain *et al.*, 2012a; Manjula *et al.*, 2013). The presence of bioactive compounds in the root extract of *C. ternatea* like the pentacyclic triterpenoid, taraxerol was allegedly correlated with the memory enhancing properties of the plant (Vasisht *et al.*, 2016; Damodaran *et al.* 2018). Furthermore, other compounds such as (Z)-9,17-octadecadienal and n-hexadecanoic was previously isolated from *C. ternatea* root extract which were tested to be effective against neurological disorder (Margret *et al.*, 2015). It was also reported that the root extracts of *C. ternatea* exhibited significant nootropic, memory retention and anti-depression effects as well as being suggested as an alternative treatment for neurodegenerative diseases (Taranalli & Cheeramkuzhy, 2000; Rai et al., 2002; Margret *et al.*, 2015). Hence, *in vitro* root culture of *C. ternatea* could be established

as a sustainable source to produce useful medicinal secondary metabolites with the goal to treat neurodegenerative diseases.

In order to acquire adequate volume of valuable medicinal compounds especially from plants to meet the demand in the pharmaceutical industries, a mass quantities of plant materials are required. The amount of these metabolites produced in the wild or cultivated needed much plant mass which results in a minute amount of secondary metabolites being extracted. Moreover, these amount again will vary in terms of cultivation methods and the exposure to the growing conditions which will result in low product standardization and cost inefficiency. *In vitro* plant tissue or organ cultures can be established as a sustainable alternative to supply to the needs for plant secondary metabolites at consistent and efficient manner, targeting specific plant organs that produces the specific metabolites. *In vitro* root cultures have been previously established for many other medicinal herbs such as *Psoralea corylifolia*, *Eleutherococcus koreanum*, *Hemarthria compressa*, *Panax ginseng* and many more for the production of natural bioactive compounds produced in the root cells (Baskaran & Jayabalan, 2009; Lee *et al.*, 2011a; Yan *et al.*, 2014; Murthy & Paek, 2016). High proliferation rate and accumulation of active secondary metabolites of the *in vitro* root culture can be achieved by manipulating the culture condition as well as phytohormones supplementation. *In vitro* root culture also offers the production of more stable bioactive compounds as metabolism is regulated in a tissue-specific manner and differentiated organ cultures are established with designated biosynthetic ability (Rao & Ravishankar, 2002; Murthy *et al.*, 2008).

Plants exhibit great regenerative capacities in facing and confronting the biotic and abiotic stresses in the environment as a result of their sessile state of development (Ashraf *et al.*, 2018; Perez-Garcia & Moreno-Risueno, 2018). It is hypothesized that

the other parts of a plant such as hypocotyl and cotyledon can be induced by using plant growth regulator (PGRs) to produce adventitious root which will then express similar medicinal value as the plant grown in its natural niche. The current study aims to utilize different explants from *in vitro C. ternatea* L. var. *ternatea* (blue colour, single-flowered) seedlings to assess the regeneration of adventitious roots and the establishment of adventitious root culture in solid and liquid media of this plant for memory enhancing studies on neuroprotective mechanism. The objectives of the current study include:

1. To investigate the effects of different auxins on inducing the adventitious root production of *C. ternatea;*
2. To establish root cultures in liquid media in producing adventitious root of *C. ternatea;*
3. To extract and identify the presence of secondary metabolites in the adventitious root culture of *C. ternatea* using gas chromatography-mass spectrometry (GC-MS), and
4. To determine the memory enhancing effect of the ethanolic root extracts of *C. ternatea* adventitious root culture using Ellman's method.

CHAPTER 2

LITERATURE REVIEW

2.1 *Clitoria ternatea* L.

2.1.1 Characteristics, morphology and distribution

Clitoria ternatea L. is belonging to the Fabaceae family which is the third largest flowering plant family and the second largest family of medicinal plants (Gao *et al.*, 2010; Aeron *et al.*, 2014). It is a leguminous perennial climber native to Africa and is abundantly found in India, Philippines, Africa, Australia and Malaysia. Commonly known as Butterfly pea or Asian pigeonwings, *C. ternatea* is also grown as an ornamental plant as it blooms flowers with vivid colours ranging from blue, mauve to white (Figure 2.1). These flowers are usually solitary, axillary or paired that bloom throughout the year (Gupta *et al.*, 2010). It is traditionally used as a natural food colouring and as tea in Asian culture. In Malaysia, the flower of the plant is commonly used to impart vibrant blue colour for traditional Malay or Nyonya cuisines such as nasi kerabu, pulut inti and pulut taitai. The stems of *C. ternatea* is fine twinning and slightly pubescent. The plant bears pinnate leaves with 5 to 7 leaflets which are usually ovate, elliptic or orbicular in shape.

The propagation of *C. ternatea* is generally through seeds and it is self-pollinated with ploidy level of 2n=16 (Morris, 2009; Gupta *et al.*, 2010). The pods of *C. ternatea* are flat and linear which are around 5-10 cm in length with 6-10 brownish or black colour seeds (Mukherjee *et al.*, 2008; Zingare *et al.*, 2013) (Figure 2.1 B&C). Matured pods will explode releasing seeds for propagation and the species exhibits epigeal germination. Owing to its vigorous survival to compete with weeds once it is established, *C. ternatea* is grown as a companion crop in Africa (Morris, 2009). This

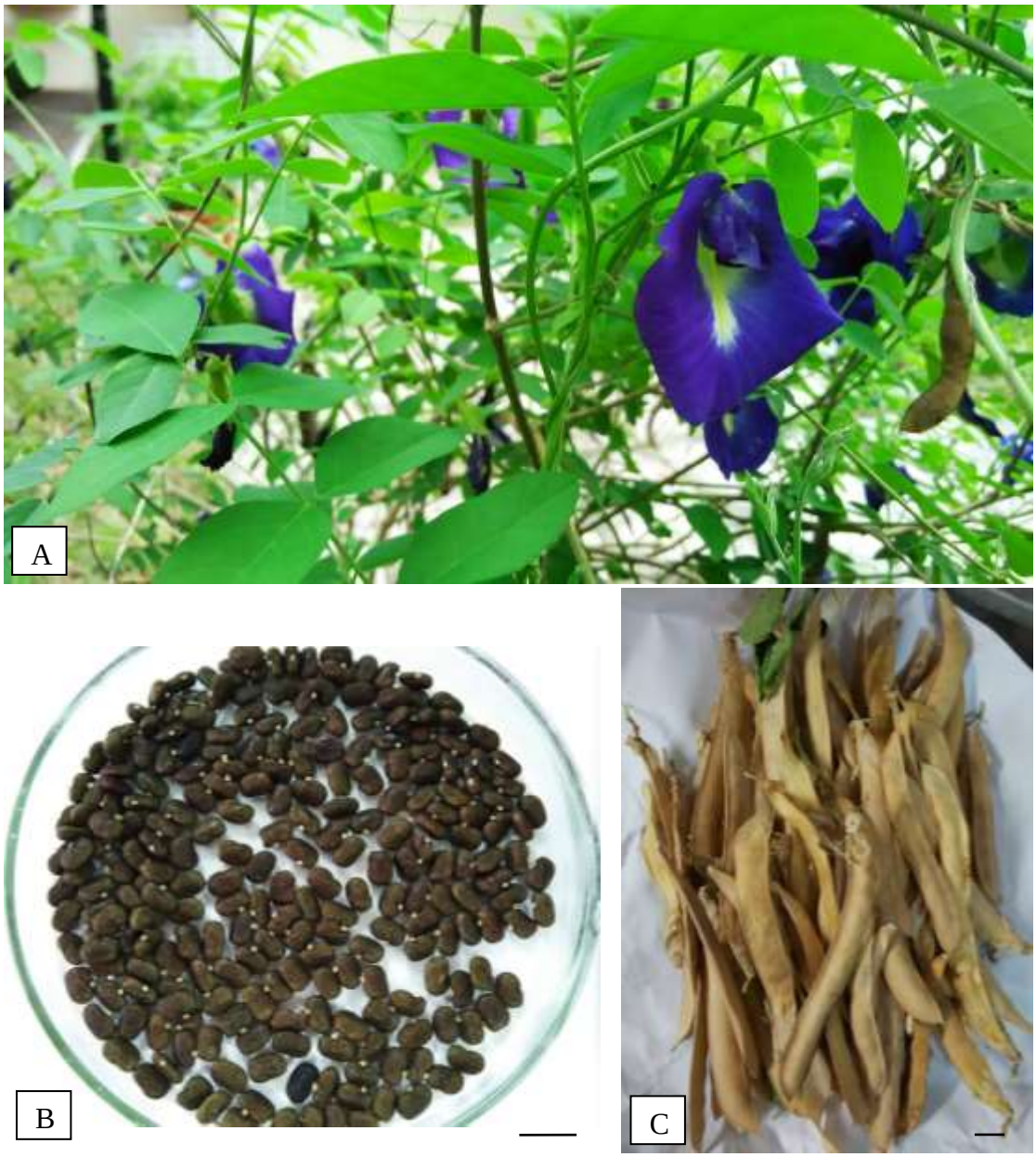

Figure 2.1: Images of *C. ternatea* L. and its structures. A) The plant of *C. ternatea* L. with blooming flowers, B) *C. ternatea* L. seeds and C) dried seed pods of the plant. (Bar=1 cm).

evergreen twinning herb is an ideal forage legume preferred by livestock and useful as cover crops (Murkhejee *et al.*, 2008). *C. ternatea* has excellent tolerance toward a wide range of soil types (pH 5.5-8.9) including calcareous soil (Gomez & Kalamani, 2003; Morris, 2009). It is managed to tolerant with extended rainfall and drought as well.

Furthermore, *C. ternatea* can be used as a cover crop because of its ability to fix nitrogen and enrich the soil (Gupta *et al*., 2010; Mohamed & Taha, 2011). *C. ternatea* forms branches of sturdy taproot with an abundance of thin lateral roots (Mukherjee *et al.*, 2008). The woody root of *C. ternatea* is whitish in colour and taste bitter. There are around 10 layers of polygonal elongated thin-walled cells containing starch grains that made up the cortex of the root (Mukherjee *et al.*, 2008). *C. ternatea* is deemed as a fertilizing legume that can contribute to over 200 kg fixed nitrogen per hectare per year along with *Leucaena leucocephala*, rendered it a useful species for revegetation and restoring soil fertility (Gomez & Kalamani, 2003; Dighe *et al.*, 2009; López-López *et al.*, 2012). This is primarily due to the presence of nitrogen fixing rhizobia found in the root nodule of the legume. A study performed by Duangkhet *et al.* (2018) reported that the two strains of nitrogen-fixing rhizobium, *Bradyrhizobium elkanii* and *Bradyrhizobium japonicum* were isolated from *C. ternatea* root nodules in Thailand.

2.1.2 Medicinal properties of *C. ternatea* L.

2.1.2(a) Traditional applications

C. ternatea is widely used as traditional medicine especially by Asians due to the variety of medicinal properties it possessed. In the Indian Ayurveda medicine, the root, leaves, flowers and seeds of the plant are used as remedies for a wide range of ailments. The roots and leaves of *C. ternatea* exhibit anthelmintic property and are used to treat infections, body aches and urinogenital disorders (Mukherjee *et al.*, 2008; Dighe *et al.*, 2009). The juice of the flower is employed as an antidote for animal stings such as snake and scorpion (Al-Snafi, 2016). The seeds are used in swollen joints and as laxative, which also exhibit slight emetic effect (Mukherjee *et al.*, 2008; Al-Snafi, 2016).

Besides, the root of *C. ternatea* is also used in the treatment of fever, constipation, indigestion, inflammation, leprosy, ulcer, asthma and arthritis (Mukherjee *et al*., 2008; Dighe *et l*., 2009). The uptake of the root along with honey or ghee is given to children to boost mental faculties. As mentioned by Dighe *et al.* (2009), the decoction of the root is prescribed for rheumatism and otalgia and is also used as emmenagogue to promote menstruation in Cuba.

2.1.2(b) Memory enhancing properties

A review done by Dighe *et al.* (2009) stated that the methanolic extract of *C. ternatea* roots possessed numerous medicinal effects including nootropic, antidepressant, anti-inflammation, anticonvulsant and anxiolytic. Being known as Aparajita or Shankhpushpi, *C. ternatea* as a ‘tonic of the nerves’ is claimed to promote memory and learning performance (Dighe *et al.*, 2009). It was reported that the root of the plant is a useful ingredient in the ancient Ayurvedic medicinal system (Taranalli

& Cheeramkuzhy, 2000; Mukherjee *et al*., 2008; Sethiya *et al*., 2009). The extracts of *C. ternatea* is added in the Indian traditional medicine, 'Medhya Rasayana' that is deemed as a nootropic recipe (Mukherjee *et al.*, 2008).

Taranalli and Cheeramkuzhy (2000) reported that the effect of *C. ternatea* root extract (CTR) in the retention of memory on rats subjected to electroshock-induced amnesia was linked to the elevation of the levels of the neurotransmitter, acetylcholine and enzyme choline acetyltransferase (ChAT) in the brain. Besides, the alcoholic extract of roots showed better anti-amnesic ability in the retention of memory of electroshocked rats as compared to the extracts from aerial parts in a dose-dependent fashion (Taranalli & Cheeramkuzhy, 2000). The learning and memory enhancing effects exerted by aqueous CTR were also investigated by Rai *et al.* (2001) using neonatal rats. Improved memory retention was observed and subsequently deduced to be resulted from the increased of acetylcholine (ACh) content in rat hippocampi as well as the improved dendritic arborisation in amygdala neurons (Rai *et al.*, 2001; Rai *et al.*, 2002; Rai *et al.*, 2005). Rai *et al.* (2005) suggested that the CTR may contain compounds similar with neuronal growth factors or brain derived neurotrophic factors found in the brain that are vital for neuronal plasticity and the survival in the hippocampus or amygdala.

Rai (2010) suggested that the aqueous CTR could serve as a neurogenic growth promoter which possibly is acting in a similar fashion like FGF-2, HB-EGF and Neuregulin 1 in promoting neurogenesis. This is primarily due to its growth promoting neurogenic effect on stimulating the growth and differentiation of neural stem cells in the anterior sub-ventricular zone (aSVZ), which is known as the site of neurogenesis (Rai, 2010). Mukherjee *et al*. (2008) mentioned that CTR exerts certain influence on cholinergic activity of brain similar with pyritinol for memory retention. Furthermore,

Rai (2010) also proposed that aqueous CTR could be the remedy for disorders like cognitive disorders, dementia or Alzheimer's disease as they discovered that 200 ng/ml of aqueous CTR had the potential to boost the production of neurons in aSVZ neurosphere as well as enhanced neuronal survival.

Malik *et al.* (2011) investigated the effects of the aqueous methanol extracts of *C. ternatea*, *Convolvulus phuricaulis* Chois. and *Evolvulus alsinoides* L. which are well-known as Shankpushpi in the Ayurvedic literature as a nootropic and memory enhancing medication. Their study showed that *C. ternatea* at the concentration of 100 mg/kg p.o. (administered orally) exhibited maximum anxiolytic and memory enhancing activities which resulted in significant antidepressant activity at lower dose levels (Malik *et al.*, 2011). Furthermore, Margret *et al.* (2015) reported on the potential of ethanolic CTR as a remedy for neurodegenerative diseases by examining its ability in inhibiting monoamine oxidase (MAO) action which is accountable for the oxidative deamination of neurotransmitters including dopamine and serotonin in the brain. The potency of ethanolic CTR as a MAO-A inhibitor was illustrated with the presence of phytocompounds, namely n-hexadecanoic acid and (Z)-9,17-octadecadienal which were deemed to possess neuroprotective, antioxidant, anticonvulsant and antidepressant properties (Margret *et al.*, 2015). The compound 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4one successfully isolated from the ethanolic extract of *C. ternatea* also exhibited good binding affinity to MAO-A active site (Margret *et al.*, 2015).

On the other hand, Vasisht *et al.* (2016) identified the presence of norneolignans viz. clitorienolactone A and clitorienolactone B in the roots of *C. ternatea* which showed remarkable learning and memory improvement demonstrated by the decrease in the time required to escape in Morris water maze study and the

increased acetylcholinesterase (AChE) inhibitory activity. As compared to the standard drug donepezil (1 mg/kg) which exhibited 43% decrease in escape latency, clitorienolactone A (5 mg/kg) and clitorienolactone B (20 mg/kg) showed decrease of 65% and 64% respectively (Vasisht *et al.*, 2016). Results from the AChE assay using Ellman method demonstrated that clitorienolactone A and clitorienolactone B have resulted in 41% (5 mg/kg) and 49% (20 mg/kg) inhibition towards the enzyme respectively as compared to donepezil (1 kg/ml) with 42% of inhibition (Vasisht *et al.*, 2016). The efficacy of methanolic CTR in mitigating the hippocampal long-term potentiation (LTP) deficits in rat induced by chronic cerebral hypoperfusion (CCH) was determined by Damodaran *et al.* (2018) in which the CTR can facilitate the hippocampal LTP and reverse the memory retrieval deficit after the permanent bilateral occlusion of common carotid arteries (PBOCCA) induced CCH. The authors claimed that the presence of taraxerol, a pentacyclic triterpenoid found present in the extract was responsible for the memory improving ability exhibited by CTR (Damodaran *et al.*, 2018).

2.1.2(c) Anti-diabetic

Diabetes mellitus (DM) is a chronic degenerative endocrine disorder characterized by hyperglycemia and disturbance in the metabolism of carbohydrates, proteins and lipids correlated with deficiency or malfunction in the secretion or action of insulin (Daisy *et al.*, 2009; Talpate *et al.*, 2013; Hasan & Sultana, 2018). This chronic disease affects approximately 5% of the world population (Verma *et al.*, 2013). Urbanization, changing of lifestyle, obesity and consumption of energy-rich diet have shaped DM into one of the main threats for human health in 21st century (Talpate, 2013). Currently, treatments available for DM such as insulin therapy, insulin secretors

and α-glycosidase inhibitors have come across deficiencies like short shelf life, ineffectiveness following oral administration, repeated injections of insulin or synthetic drugs required and fatal hypoglycaemia with excess dosage (Daisy and Rajathi, 2009; Talpate *et al.*, 2013). Moreover, Verma *et al.* (2013) stated that most oral antidiabetic drugs only offer symptomatic relief aside from effectively treating diabetes. Various plant extracts have discovered with bioactive compounds that exert antihyperglycemic effect more efficient than oral hypoglycemic agents used in clinical therapy (Gunjan *et al.*, 2010).

Mathada *et al.* (2012) suggested that juvenile diabetes has impact on the cognitive function as the postnatal development of the brain can be affected by the hyperglycemic condition caused by excessive glucose circulating in the blood stream due to the scarcity of insulin. Microscopic assessment of the pancreatic tissue of juvenile Wistar rats which were given intra peritoneal streptozotocin injection of 60 mg/kg body weight (b.w.) showed tissue fragmentation. Serious neurodegeneration was also detected in the brain cortex with the deflated cells of hippocampal CA 3 showing vacuolization and chromotolysis of cytoplasm (Mathada *et al.*, 2012). However, treatment of 100 mg/kg b.w. *C. ternatea* root extract to the juvenile diabetic rats displayed good integrity in the pancreatic tissue with reduced cell hypertrophy and islet cells can be observed (Mathada *et al.*, 2012). Cytoplasm was noticed without neuronal shrinkage and vibrant homogenous arrangement of hippocampal pyramidal cells in the CA 3 area can be detected (Mathada *et al.*, 2012). This indicated that the *C. ternatea* root extract is managed to restore the cognitive functions of the brain resulted by the complications of diabetes.

2.1.2(d) Anti-cancer

Cancer is a major concern of health for human in the 20th century which is a result from hyperproliferation disorder of cells involving dysregulation of apoptosis, transformation and proliferation (Sharma & Rawal, 2012). Cancer has the latency to spread and invade within the body in the form of tumour. There are factors that contribute to the formation of tumour or cancer, such as radiation, pollution, smoking, chemicals and so on (Iqbal *et al.*, 2017). It was estimated by the World Health Organization (WHO) that cancer might result in approximately 12 million deaths by the year 2030 (Farooqui *et al.*, 2011). Studies have been done to discover phytochemicals in plant or plant-derived materials with anticancer activity that could act specifically on tumour cells without inducing severe side effects or affecting normal cells (Iqbal *et al.*, 2017).

Ayurvedic practitioners since the ancient time have employed the decoction of *C. ternatea* leaves as a purification prescription for patients after surgical removal of tumour (Balachandran & Govindarajan, 2005). Ramaswamy *et al.* (2011) described the cytotoxic properties of ethanolic extract of *C. ternatea* leaf with half maximal inhibitory concentration (IC_{50}) of 305 µg/ml. Besides, Jacob and Latha (2012) stated that methanolic seed extract of *C. ternatea* generated significant effects on reducing tumour volume, viable cell count and packed cell volume when compared with the control group that had received Dalton's lymphoma ascites tumour intraperitoneally.

Swain *et al.* (2012a) claimed that taraxerol is an essential pentacyclic triterpenoid that possesses a wide range of medicinal properties including anticancer. The high-performance thin layer chromatography (HPTLC) examination on the extract obtained from transformed root culture of *C. terneatea* demonstrated the presence of taraxerol (Swain *et al.*, 2012a). Sen *et al.* (2013) also reported remarkable

chemosensitizing and anticancer activities detected in the presence of cyclotides isolated from the flower and seed of *C. ternatea* against paclitaxel-resistant lung cancer cells with reduced IC_{50} values recorded.

2.1.2(e) Hepatoprotective

Liver is a vital regulatory organ in the body that plays crucial roles in body homeostasis, metabolism, storage, secretion, and most importantly, detoxification of waste metabolites and toxic compounds. Hepatic disease is referred to the impairment of the cells, tissues, structure or liver function which usually caused by excessive consumption of chemicals or drugs including alcohol and paracetamol, bacteria or virus infection and autoimmune diseases (Madrigal-Santillán *et al.*, 2014). This has been a major concern for public health and it is an ongoing process to discover effective medicines and pharmaceutical alternatives that could provide protection to the organ and the regeneration of hepatic cells.

The hepatoprotective activities of the seed and root of *C. ternatea* against acetaminophen- and carbon tetrachloride (CCl_4)-intoxicated were tested by Solanki and Jain (2011) using Wistar albino rats. By assessing the level of liver serum marker enzymes including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin in the blood serum of treated rats which will elevate in response to the membrane lipid peroxidation after induced hepatic damage, the hepatoprotective activity was evaluated (Solanki and Jain, 2011). They claimed that 500 mg/kg b.w. of hydroalcoholic extracts of *C. ternatea* seeds demonstrated significant hepatoprotective potential which substantially reduced the levels of AST, ALT, ALP and total bilirubin in the blood serum of both acetaminophen- and CCl_4-intoxicated rats (Solanki & Jain, 2011). However, the root

extract only showed hepatoprotective activity in CCl_4-intoxicated rats. According to Solanki and Jain (2011), the hepatoprotective effects exerted by *C. ternatea* could be associated with its abilities in inhibiting lipid peroxidation and mast cells infiltration as well as being a strong antioxidant observed through histopathological studies of liver tissues.

2.1.2(f) Anti-asthmatic

C. ternatea is recognized as a useful material employed in the treatment of asthma and bronchitis in the traditional medicine systems. The inflammatory disorder of respiratory tracts characterized by bronchial hyper responsiveness, airway inflammation and airway obstruction which generally known as bronchial asthma which been reported to affect approximately 7-10% of the world population (Chauhan *et al.*, 2012a). According to Savithramma *et al.* (2007), *C. ternatea* root was utilized in the treatment of asthma in Andhra Pradesh, India through the intake of the decoction of 100 mg powdered root twice a day orally.

Taur and Patil (2011) evaluated the anti-asthmatic effect of the ethanolic CTR using albino mice and wistar rats as animal models. The rats were subjected to milk induced eosinophilia and leucocytosis. Increased leucocytes and eosinophils counts will trigger hypersensitivity reaction in asthmatic patient. Their results indicated that the ethanolic CTR has the potential in suppressing the type 1 hypersensitivity reaction by effectively reducing the leucocytes and eosinophils count found in the rats pre-treated with ethanolic CTR at concentration of 100-150 mg/kg injected intraperitoneally (i.p.) comparable to the positive control, 50 mg/kg of dexamethasone (Taur & Patil, 2011). In the examination of anti-asthmatic effect using egg albumin induced mast cell degranulations in mice, ethanolic CTR demonstrated protection to

the mast cells from degranulation where 150 mg/kg i.p. resulted in 58.33% of inhibition on the par with 69.59% inhibition produced by sodium chromoglycate, the positive control (Taur & Patil, 2011).

In addition, Chauhan *et al.* (2012a) reported that the ethanolic CTR displayed significant bronchospasmolytic activity that protect the respiratory tract against histamine-induced bronchospasm at concentration of 400 mg/kg p.o. (47.45%). Furthermore, the researchers claimed that the aqueous extract of *C. ternatea* root also exhibited bronchodilating effect in resisting histamine-induced bronchoconstriction in rats and capable of minimizing the bronchial hyperreactivity (Chauhan *et al.*, 2012a).

2.1.2(g) Antipyretic

Regulated elevation of body temperature in response to the increase of hypothalamic set point after experiencing an infectious or aseptic stimulus is known as fever (Aronoff & Neilson, 2001). The presence of pyrogens triggers the release of mediators, particularly prostaglandin E_2 (PGE_2) in the preoptic nuclei of anterior hypothalamus which will interfere with the firing rate of neurons in the hypothalamus that control the thermoregulation (Aronoff & Neilson, 2001). According to a review performed by Aronoff and Neilson (2001), antipyretics are characterized by drugs that exhibit effects in inhibiting cyclooxygenase and lower the level of PGE_2 in hypothalamus as well as possess the abilities to boost anti-inflammatory signals at the site of injury and decrease pro-inflammatory mediators within the brain.

Allergy reactions might arise with the use of commonly prescribed antipyretics including aspirin, acetaminophen and ibuprofen in some patients especially asthmatic patients. Herbal medicines have been widely explored for efficient pharmaceutical properties with fewer side effects. Parimaladevi *et al.* (2003) examined the antipyretic

effect of methanolic CTR by exposing Wistar rats to yeast-induced pyrexia where they found out that oral administration of methanolic CTR at 200 and 400 mg/kg significantly reduced the yeast-induced fever to 37.9±0.05 °C and 37.4±0.04 °C respectively after 23 hours which were comparable to the effects exerted by 150 mg/kg of paracetamol (37.2±0.03 °C).

2.1.2(h) Anti-inflammatory

Inflammation is a body defence response where the body tissues react in a way to remove or avoid the spreading of harmful agents as well as necrosed cells and tissues in response to injuries or infections. Common symptoms associated with inflammation are pain and fever (Patil & Patil, 2015). Nonsteroidal anti-inflammatory drugs (NSAID) are drugs that are employed to reduce pain, fever and inflammation including aspirin, phenylbutazone, indomethacin and so forth. The side effects concerning the usage of NSAID are specific to different drugs which could give rise to kidney disease, heart attack and gastrointestinal ulcers. Ayurveda medicines system registered a variety of herbal medicines that contribute to the fundamental health care with lesser apprehensions about its side effects.

Parimaladevi *et al.* (2003) assessed the anti-inflammatory activity of methanolic CTR utilizing carrageenin-induced rat paw oedema and acetic acid-induced vascular permeability in rats. They claimed that 400 mg/kg p.o. of methanolic CTR generated 31.8% of inhibition to the carrageenin-induced oedema that was comparable to the result produced by 20 mg/kg of diclofenac, a standard NSAID (Parimaladevi *et al*., 2003). Similarly, 200 and 400 mg/kg p.o. of methanolic CTR reduced the peritoneal inflammation by 35.94% and 55.11% in acetic acid-induced

cascular permeability as compared to 60.55% inhibition by the diclofenac (Parimaladevi *et al*., 2003).

Nair *et al.* (2015) reported that the ternatin anthocyanins and quercetin glycosides isolated from the blue flower petals of *C. ternatea* exhibited anti-inflammatory effect on lipopolysaccharide (LPS)-induced inflammation by the suppression of excessive pro-inflammatory mediator production from macrophage cells. They also suggested it as a potential remedy for chronic inflammatory diseases. On the other hand, the methanolic extracts of the leaf of *C. ternatea* were reported with significant anti-inflammatory activity assessed using carrageenin-induced paw oedema in rats. The oral administration of 500 mg/kg of the methanolic leaf extract demonstrated 66.66% of oedema inhibition in comparison to 70.58% of 10mg/kg p.o. diflofenec. These studies revealed that the leaves and roots of *C. ternatea* possessed remarkable anti-inflammatory activity which was postulated to be related to its ability in inhibiting the inflammatory mediators such as prostaglandin and cyclooxygenase (Patil & Patil, 2015).

2.2 Tissue culture of *C. ternatea* L.

Nowadays, a great number of pharmaceutical products are derived from natural plant materials. The over-exploitation activities of natural plant resources to meet the increasing demands of natural herbs and the lack of preservation might lead to the loss of plant populations or even extinction. Plant cell and tissue culture has evolved as a prevailing biotechnological practice in assisting the development of plants that are vulnerable to grow in nature additionally ensuring a sustainable conservation of the biodiversity. The application of this technique involves the manipulation and optimization of the growth conditions suitable to the plant. This includes culture

medium, light, temperature and humidity which assure healthy development of superior plant materials despite the concerns of geographical and climate factors.

Previous studies on tissue culture of *C. ternatea* is summarized in Table 2.1. *In vitro* shoot multiplication of *C. ternatea* was established by Rout (2005) with the supplementation of 8.9 µM of 6-benzylaminopurine (BAP) in Murashige and Skoog (MS) medium and the highest rooting frequency (90.2±1.6%) was reported when ½ MS medium fortified with 1.34µM of α-naphthalene acetic acid (NAA). On the other hand, *in vitro* micropropagation of *C. ternatea* conducted by Shahzad *et al.* (2007) using excised root segments as plant material is believed to be highly regenerative. Shahzad and colleagues (2007) reported that direct shoot bud initiation was observed from explants growth on semisolid MS medium supplemented with 6-benzyladenine (BA). Moreover, addition of certain concentration of NAA in MS medium containing BA has further improved the regenerative potential of the explant. This indicated that root explant has the potential to be a starting material for efficient *in vitro* micropropagation of *C. ternatea*.

An established micropropagation protocol of *C. ternatea* reported by Pandeya *et al.* (2010) revealed that cotyledonary node, shoot tip and nodal explants exhibited great potential of generating multiple shoots of the plant in MS medium augmented with 2.0 mg/L BAP. In addition, 0.5 mg/L gibberellic acid (GA_3) was found beneficial for shoot elongation and the procedure of immersing the basal cut end of the elongated shoots into 250 mg/L indole-3-butyric acid (IBA) for half an hour facilitated the *ex vitro* rooting of micropropagated shoots of *C. ternatea* (Pandeya *et al.*, 2010). Ismail *et al.* (2012) mentioned that the utilization of nodal explant for direct shoot multiplication of *C. ternatea* is more effective as compared to cotyledonary node and

Table 2.1: Tissue culture studies of *C. ternatea* L.

Explants/ Starting materials	Culture condition/ PGRs	Results				References
		Shooting	Rooting	Days to rooting	Callus	
De-coated seed	MS basal medium, Kin, IBA, IAA	MS + 0.5 mg/L Kin	MS + 0.5 mg/L IBA	15-20	MS + 0.1 mg/L Kin	Lakshmanan & Dhanalakshmi (1990)
Nodal	MS basal medium, BA, Kin, IAA, NAA	MS + 8.9 µM BA, 1.34 µM NAA	MS + 1.34 µM NAA	7-8	-	Rout (2004, 2005)
Cotyledonary node	MS & ½ MS basal medium, ZR, BA, TDZ, IPA, NAA	MS + 1.0 mg/L BA	½ MS + 0.25 mg/L IBA	8-10	-	Barik *et al.*, (2007)
Root segment	MS & ½ MS basal medium, NAA, IAA, 2,4-D, BA, Kin	MS + 20 µM BA, 2.0 µM NAA	½ MS + 5 µM IBA	7-14	MS + 5 µM BA, 10 µM 2,4-D	Shahzad *et al.*, (2007)
Cotyledonary node	MS basal medium, BA, Kin, 2-iP, NAA	MS + 2.5 µM BA	½ MS + 1.0 µM IBA	7-14	-	Mukhtar *et al.* (2010)
Shoot tip, node, cotyledonary node	MS, BAP, NAA	MS + 2.0 mg/L BAP	250 mg/L IBA (Dipping in solution for ½ hour)	14-21	-	Pandeya *et al.*, (2010)
Cotyledonary node	MS & B5 basal medium, BAP, Kin, TDZ	MS + 1.5 mg/L BAP	500 mg/L IBA (Dipping in solution for 5 minutes)		-	Singh & Tiwari (2010).
Axillary bud	MS, BAP, Kin, NAA, IAA	11 M BAP + 1.34 M NAA	MS + 3 M NAA	7-14	-	Mhaskar *et ai.*, (2011)

Table 2.1 continued

Leaf	DKW basal medium, NAA, BAP	DKW + 1.0 mg/L BAP	DKW + 2.0 mg/L NAA	7-14	DKW + 2.0 mg/L BAP + 1.0 mg/L NAA	Mohamed & Taha (2011)
De-coated seed, leaf, node, petiole	MS & ½ MS basal medium, 2,4-D, Kin, BA, IAA, NAA	MS + 0.75 mg/L BA + 0.5 mg/L IAA	½ MS + 1.0 mg/L NAA	7-14	MS + 1.0 mg/L 2,4-D + 0.5 mg/L Kin	Arumugam & Panneerselvam (2012).
Shoot tip, node, cotyledonary node	MS & ½ MS basal medium, BA, Kin, IBA	MS + 5 µM BA	½ MS + 2.0 µM IBA	7-14	-	Ismail *et al.*, (2012)
Hairy root culture	Liquid MS basal medium, IBA		Liquid MS + 0.25 mg/L IBA	25-30	-	Swain *et al.*, (2012b)
Nodal	MS basal medium, BAP, NAA, 2,4-D	MS + 2.5 mg/L BAP + 0.5 mg/L NAA	MS + 0.5 mg/L NAA	7-14	MS + 2,4-D (0.5 – 2.5 mg/L)	Madhu (2013)

shoot tip on MS medium fortified with 5.0 µM of BA which resulted in 90% of response. The *in vitro* root induction of the elongated shoots was established on half strength MS medium supplemented with 2.0 µM of IBA resulted with 80% of rooting frequency (Ismail *et al.*, 2012).

On the other hand, Barik *et al.* (2007) investigated the *in vitro* axillary shoot proliferation of *C. ternatea* by employing cotyledonary node explants obtained from axenic seedlings. They found out that 1.0 mg/L BA fortified MS medium produced significantly higher percentage of shoot proliferation (96.6%) as well as generated the greatest number of shoots per explant (5.2±0.22) and longest shoot length (6.4±0.13 cm) as compared to the supplementation of other phytohormones tested like zeatin riboside and thidiazuron (Barik *et al.*, 2007). Rooting of the *in vitro* proliferated plantlets was best induced in half strength MS medium added with 0.25 mg/L IBA (1.42 µM) and subsequently acclimatized. Barik *et al.* (2007) stated that half strength MS medium without any supplementation of phytohormones has failed to induce rhizogenesis from the regenerated shoots indicated that *in vitro* rooting of *C. ternatea* required the presence of auxins. On the other hand, Mukhtar *et al.* (2010) discovered that the use of cotyledonary nodes as starting material for shoot regeneration of *C. ternatea* and in treatment of MS medium supplemented with 2.5 µM BA resulted in significantly higher shoot regeneration rate (85.2±1.58%), number of shoot formed (9.8±0.86) and shoot length (11.0±0.45 cm). Combination of 2.5 µM BA with 1.0 µM NAA substantially promoted shoot proliferation of leaf explants in MS medium (Mukhtar *et al.*, 2010). In line with Barik *et al.* (2007), Mukhtar *et al.* (2010) reported that 1.5 µM of IBA augmented half strength MS medium was the optimal rooting medium for the microshoots generated from cotyledonary node explants *in vitro*.

Additionally, Mhaskar *et al.* (2011) described the effectiveness of using axillary bud explant for *in vitro* regeneration of *C. ternatea* in which the highest percentage of shoot responded and number of shoots generated were found in MS medium fortified with 11 µM BAP in combination with 1.34 µM NAA. As for *in vitro* root induction of the regenerated *C. ternatea* shoots, the addition of 3 µM NAA in MS medium resulted in 80% of rhizogenesis frequency after 4 weeks of culture and 7-8 weeks old rooted plantlets were successfully acclimatized after 21 days in pots (Mhaskar *et al.*, 2011). Furthermore, Mohamed and Taha (2011) studied the regeneration potential of *C. ternatea* leaf explants by culturing the *in vitro* generated leaves on Driver and Kuniyuki (DKW) medium augmented with different concentration of NAA and BAP either singly or in combination. Results showed that supplementation of BAP alone induced shoot formation whereas addition of NAA induced root formation from the leaf explants (Mohamed and Taha, 2011).

Apart from these, plant regeneration protocol conducted by Kumar and Thomas (2012) through *in vitro* somatic embryogenesis using embryonic callus produced by cotyledon explants of *C. ternatea* has proven to be highly effective. The presence of 2,4-D in MS medium successfully induced callus formation from cotyledon explants of *C. ternatea* and further sub-culturing of the callus on MS medium supplemented with 2.0 mg/L BA and 0.5 mg/L NAA yielded remarkably higher somatic embryo induction rate (61%) in comparison to the MS medium comprised of the combination of kinetin with NAA (Kumar & Thomas, 2012). In addition, Kumar and Thomas (2012) claimed that 3 mg/L of abscisic acid (ABA) exhibited significant effect on the embryo induction when added in MS medium containing 2.0 mg/L BA and 0.2 mg/L NAA which generated the highest frequency of embryogenic response (83%).

2.3 Organogenesis of root

Formation of new organs from undifferentiated cells is defined as organogenesis (Lavenus *et al.*, 2013). Most organs in plant are formed during postembryonic developmental stage and *in vitro* organogenesis of plants is achievable through tissue culture techniques using different explants owing to the totipotency of plant cells. According to Sugiyama (1999), *in vitro* organogenesis is in regard to factors such as phytohormone sensitivity, organization of cell division for the development of specific organ meristems or primordia and organogenic competence of the differentiated cells to dedifferentiate. *In vitro* organogenesis is depending on the effect of exogenous PGRs on three prominent phases; dedifferentiation of cells to attain organogenic competence, determination of the fate of dedifferentiated cells for specific organ formation and the morphogenesis of organ to proceed independently in response to exogeneous phytohormones (Sugiyama, 1999).

The major functions of root in plant include anchoring the plant into the soil, minerals and water absorption as well as storing photoassimilates which are performed by the vascular tissues (xylem and phloem) present in the root system (Bellini *et al.*, 2014). Primary root of a plant is originated from the radicle of an embryo during germination (Bellini *et al.*, 2014). Lavenus *et al.* (2013) studied the organization of root using *Arabidopsis thaliana* stated that the root composed of central vascular system, single-layered pericycle, endodermis, cortex and epidermis are developed by the root apical meristem (RAM) derived from the basal part of the embryo. Precise cell division regulation and identity patterning are the keys to root meristem organogenesis and histogenesis (Lavenus *et al.*, 2013). Lateral roots emerge endogenously from the mature part of the root above RAM and expand the absorptive surface of the plant (Jackson, 1986; Lavenus *et al.* 2013).